Chemical Translations

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TRANSLATION FROM *German*

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Pharmaceutical Preparations for Regulating the Release of Insulin by Influencing the beta-Cell of the Pancreatic Islets

as submitted to me in the

German language;

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FEDERAL REPUBLIC OF GERMANY

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Priority Document Relating to the Filing of a Patent Application

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The attached pages are a true and accurate reproduction of the original documents filed with this Patent Application.

Munich, July 7, 2004
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for The President
Signed: Schäfer

PHARMACEUTICAL PREPARATIONS FOR REGULATING THE RELEASE OF INSULIN BY INFLUENCING THE β-CELL OF THE PANCREATIC ISLETS OF LANGERHANS

The invention relates to the use of melatonin and/or the chemically modified derivatives thereof for making pharmaceutical preparations for the regulation of insulin release by influencing the β-cell of the pancreatic islets through a melatonin-specific receptor.

Melatonin (N-acetyl-5-methoxytryptamine), an indolamine, is a hormone of the pineal gland which was also found in the retina, in Harder's gland of rodents and in the enterochromaffin cells. It is used, among other things, to combat insomnia (sedative implications), reduce jet lag problems after intercontinental flights and synchronization problems arising from short-term changes in work schedule (shift work), to protect cells from free radicals (particularly hydroxyl radicals), to retard tumor growth, to prevent cataracts as well as to prolong life (S.F. Pang et al., Recent development of pineal melatonin and its receptors in humans. In: P.L. Tang et al., (eds), Melatonin: A Universal Photoperiodic Signal with Diverse Actions, Front. Horm. Res. 21:133-146, 1996).

Melatonin plays a critical role in the regulation of circadian rhythms. For example, it synchronizes the free-running sleep-wake cycle in blind persons. The photically-controlled neural influx (catecholamine influx) is converted in the pineal gland into a hormonal signal (melatonin). The pineal gland acts as a neuroendocrine "translator" and by increasing nocturnal melatonin secretion provides information about the relationship between the light and the dark periods in the course of the day (clock function) and about its changes in the course of the year (calendar function).

In mammals - in contrast to birds - the regulation of the circadian rhythm, however, does not take place directly in the pineal gland, but in a hypothalamic nuclear region, the suprachiasmatic nuclei (SCN). These nuclei play a critical role as primary "circadian pacemakers" in the generation of circadian rhythms in mammals (S. Reuss, Components and connections of the circadian timing system in mammals, Cell Tissue Res. 285: 353-378, 1996).

Especially important for the understanding of the functional interactions between this hypothalamic nucleus and the pineal gland was the detection of melatonin receptors in SCN indicating a functional interaction between the two structures (V.M. Cassone, Melatonin and suprachiasmatic nucleus. In: D.C. Klein et al. (eds.), Suprachiasmatic nucleus, The mind's clock. Oxford University Press 1991, 309-323).

The fact that, besides a multiplicity of other functional features, the density of melatonin receptors in SCN is increased during the day, whereas in contrast to this, physiological, biochemical and morphological studies have indicated an increase in activity of the pineal gland during the night, is consistent with an inhibitory effect of melatonin on the SCN as a time-related, fine-regulatory instrument (D.R.

Weaver et al., Localization of melatonin receptors in mammalian brain. In: D.C. Klein et al. (eds.), Suprachiasmatic nucleus, the Mind's clock, Oxford University Press 1991, 289-308).

Melatonin receptors were found to be present not only in the SCN, however, but also in the pars tuberalis (L.M. Williams and P.J. Morgan, Demonstration of melatonin-binding sites on the pars tuberalis of the rat, J. Endocrinol. 119: R1-R3, 1998); P.J. Morgan et al., Melatonin receptors in the ovine pars tuberalis: Characterization and autoradiographical localization, J. Neuroendocrinol. 1: 1-4, 1989), in the retina (G. Tosini and M. Menaker, Circadian rhythms in cultured mammalian retina, Science 272: 419-421, 1996), in the cerebellum (J.D. Fauteck et al., The adult human cerebellum as a target of the neuroendocrine system involved in the circadian timing, Neurosci. Lett. 179: 60-64, 1994) and more recently in peripheral tissues and organs such as the stomach, kidneys, lung, heart, testicles and others (S.F. Pang et al., Melatonin receptors in peripheral tissues: A new era of melatonin research, Biol. Signals 2: 177-180, 1993); P.J. Morgan et al., Melatonin receptors: Localization, molecular pharmacology and physiological significance, Neurochem. Int. 24: 101-146, 1994).

The possibility of influencing insulin release via the melatonin receptors in the pancreas, the Langerhans islets or the insulin-producing β-cell has thus far not been described.

At this time, different melatonin receptor subtypes are known, for example Mel_{1a}, Mel_{1b} and Mel_{1c} whose amino acid sequences and membrane structure (seven transmembrane helices) have been analyzed (S.M. Reppert et al., Melatonin receptors step into the light: Cloning and classification of subtypes, TIPS 17: 100-102, 1996). These are G-protein-coupled ($Gi_{i/o}$-coupled) receptors blocked by pertussis toxin (pertussis-sensitive G-protein). In view of these relationships, functional melatonin-receptor detections are based on, among other things, the possibility of limiting or abolishing the melatonin-induced inhibition of the forskolin-stimulated increase in cAMP by pertussis toxin (L.L. Carlson, D.R. Weaver and S.M. Reppert, Melatonin signal transduction in hamster brain: Inhibition of adenylyl cyclase by a pertussis toxin-sensitive G protein, Endocrinology 125: 2670-2676, 1989; L.L. Carlson et al., Melatonin receptors couple through a cholera toxin-sensitive mechanism to inhibit cyclic AMP in the ovine pituitary, J. Neuroendocrinol. 7: 361-369, 1995). In summary, it can be stated that in mammals the melatonin-induced inhibition of adenylate cyclase takes place through a pertussis-sensitive G-protein.

The object of the invention is to provide pharmaceutical preparations for regulating the release of insulin by influencing the β-cell of the pancreatic islets through specific receptors.

According to the invention, this objective is reached by use of melatonin and/or a chemically modified derivative thereof to make pharmaceutical preparations capable of regulating the release of insulin by influencing the β-cell of the pancreatic islets through a melatonin-specific receptor.

Surprisingly, we have now found that

- melatonin and/or chemically modified derivatives thereof realize their insulin-reducing influence through G protein-coupled membrane-bound receptors;
- through the melatonin receptor, melatonin and/or its chemically modified derivatives assume pacemaker significance, because the release of insulin from isolated pancreatic islets is at the base of circadian and ultradian rhythms;
- through the melatonin receptor, melatonin and/or chemically modified derivatives thereof in both pharmacological (5 μM) and physiological doses (0.2 nM) significantly reduce the stimulated insulin release from pancreatic islets.

Another object of the present invention are pharmaceutical preparations for oral and parenteral, including topical, rectal, subcutaneous, intravenous, intramuscular, intraperitoneal, intranasal, intravaginal, intrabuccal or sublingual, administration which besides the usual carriers and diluents contain as the active ingredient a compound claimed in Claim 1.

Suitable pharmaceutical formulations are the following:

- tablets, capsules or coated tablets with 0.01 to 200 mg of active ingredient, used orally,
- ampules with 0.01 to 200 mg of active ingredient, for subcutaneous injection,
- adhesive tape with transdermal release of 0.01 to 200 mg of active ingredient,
- subcutaneous implants with a release capacity of 0.01 to 200 mg of active ingredient,
- gels and creams with transdermal release of 0.01 to 200 mg of active ingredient,
- buccally administered systems releasing 0.01 to 200 mg of active ingredient.

The drugs of the invention are prepared in appropriate doses in the known manner with conventional solid or liquid carriers or diluents and conventional pharmaceutical-technical auxiliary agents, in accordance with the desired type of administration.

FUNCTIONAL DEMONSTRATION OF MEMBRANE-BOUND MELATONIN RECEPTORS OF THE PANCREATIC ISLET

Fig. 1 shows a graphic representation of the statistically significant drop in glucose-stimulated and KCl-stimulated insulin secretion, induced by melatonin (MT, here 5 μM) - nutrient solution containing glucose or KCl + melatonin = dark bars, in comparison with a control - nutrient solution containing glucose or KCl = light bars.

The results are statistically significant with a probability of error $p < 0.001$.

Fig. 2 shows the influence of melatonin (here 10 nM melatonin) on the forskolin-stimulated release of insulin brought about by increasing concentrations of forskolin.

The insulin-reducing influence of melatonin is significant.

It can be seen from both figures that melatonin in physiological as well as pharmacological doses reduces KCl-stimulated, glucose-stimulated and forskolin-stimulated insulin secretion. This is due to inhibition of voltage-dependent Ca^{2+} channels and/or of adenylate cyclase. Moreover, it was demonstrated in phase-response studies that melatonin, used as pacemaker, brings about a phase acceleration of circadian insulin secretion.

FUNCTIONAL DEMONSTRATION OF MEMBRANE-BOUND MELATONIN RECEPTORS OF PANCREATIC ISLETS BY USE OF GTPγS (guanosine S [gamma-thio]triphosphate)

Fig. 3 shows the influence of non-hydrolyzable GTPγS on the action of melatonin.

A comparison of data obtained for a nutrient solution containing melatonin + forskolin with a solution consisting of the nutrient solution containing melatonin + forskolin + GTPγS shows that the action of melatonin on the forskolin-stimulated release of insulin is nearly abolished by GTPγS. The reason for this is the functional blockade of the melatonin receptors by GTPγS.

These results can be interpreted as direct evidence of receptor-specific regulation of insulin release, and this for the first time constitutes proof of the existence of a functional melatonin receptor on the LANGERHANS islet.

AUTORADIOGRAPHIC DETECTION OF MEMBRANE-BOUND MELATONIN RECEPTORS OF THE PANCREATIC ISLET USING 2-$^{[125J]}$IODOMELATONIN

Fig. 4 shows an autoradiographic study of the detection of melatonin receptors on pancreatic tissue (frozen section) of neonatal rats.

The punctiform signals represent binding sites of 2-$^{[125J]}$iodomelatonin on the frozen section.

The controls show no punctiform signals.

For exact localization, the developed film plates are shown alone as well as after micromanipulation with the film plates mounted above the tissue photographed at the same time (insert bar: 400 μm).

Fig. 5 shows the quantification of the autoradiographic detection of melatonin receptors as a displacement curve reflecting the displacement of 2-$^{[125J]}$iodomelatonin from its receptor bonds by

noniodinated melatonin. The study was made by computer-assisted gray scale analysis (Optimas 2.0).

These results can be interpreted as direct evidence of the existence of melatonin-specific receptors in the LANGERHANS islet.

MOLECULAR-BIOLOGICAL DETECTION OF MEMBRANE-BOUND MELATONIN RECEPTORS OF THE PANCREATIC ISLET

Fig. 6 shows the amplified PCR product which has a length of 329 bp and represents a specific partial sequence of the melatonin receptor sequence. In this manner, the melatonin receptors in the LANGERHANS islet were detected for the first time on a molecular level.

The exact methodology of molecular-biological detection is described in the following.

1. Preparation of the Pancreatic Tissue

The pancreatic tissue used for the molecular-biological study was obtained from eight neonatal rats (male and female) and stored at -70 °C until needed for further study.

2. RNA Extraction

Total RNA was isolated from 150 mg of pancreatic tissue by the guanidine thiocyanate/LiCl method of Cathala et al., A method of isolation of intact translationally active ribonucleic acid, DNA: 329-335, 1983.

The purity and quantity of the isolated RNA were determined by photometric absorption measurement.

The quantity of isolated RNA was 150 μg.

The ratio of optical density (O.D.) at 260 nm to that at 280 nm was 1.7.

3. Establishment of a cDNA Library by Reverse Transcriptase Reaction (RT)

In the RT reaction, mRNA is reversely transcribed into complementary DNA (copy DNA, cDNA) which then serves as the starting matrix for the subsequent amplification (PCR reaction).

The cDNA synthesis was carried out according to directions provided by the manufacturer of the corresponding kit (Pharmacia-Biotech). 5 μg of RNA was used as matrix for the RT reaction. The incubation was carried out at 37 °C for 60 minutes.

4. Polymerase Chain Reaction (PCR)

The molecular-biological detection of the transcription products for the Mel_{1A} receptor was carried out by use of the PCR technique. To this end, partial sequences of the cDNA molecules of the melatonin receptors were amplified with the aid of specific oligonucleotide sequences (primers).

The resulting PCR products were detected on conventional agarose gels.

In constructing the primers, both the length (base pairs) and base composition (G/C content) of the primers and the length of the PCR products were taken into account as required by rules.

The primers were specific for a partial cDNA fragment of the rat melatonin receptor according to Reppert et al., Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses, Neuron 13: 1177-1185, 1994; cf. Accession No.: U14409.

The position of the primers covers the cDNA regions 11-33 (up primer) and 319-339 (low primer). The specific PCR product should have a length of 329 bp.

The conditions for PCR were as follows: 94 °C (1 min) - 55 °C (1 min) - 72 °C (1 min), and at the end 15 min at 72 °C.

Forty cycles were carried out.

The PCR product was investigated electrophoretically in a 2.5% agarose gel (plus ethidium bromide). The length standard was a 100 bp DNA standard.

Running time was 90 min at 50 volts in a standard electrophoresis buffer.

PATENT CLAIMS

1. Use of melatonin and/or of a chemically modified derivative thereof for making pharmaceutical preparations for the regulation of insulin release by influencing the β-cell of the pancreatic islets through a melatonin-specific receptor.

SUMMARY

The present invention relates to the use of melatonin and/or a chemically modified derivative thereof for making pharmaceutical preparations for the regulation of insulin release by influencing the β-cell of the pancreatic islets through a melatonin-specific receptor.

Surprisingly, we have found that melatonin and/or chemically modified derivatives thereof, when used according to the invention,

- realize their insulin-reducing influence through G- protein-coupled membrane-bound receptors;
- through the melatonin receptor assume pacemaker significance, because the release of insulin from isolated pancreatic islets underlies the circadian and ultradian rhythms;
- through the melatonin receptor, in pharmacological (5 μm) as well as in physiological doses (0.2 nm), reduce the stimulated release of insulin from pancreatic islets in statistically significant manner.

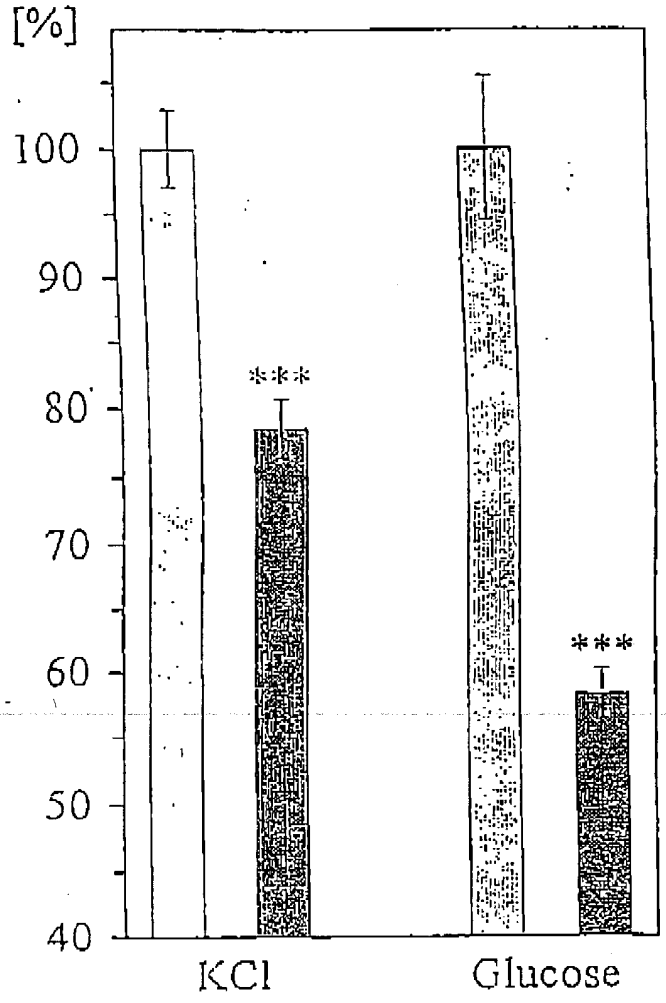

Figure 1

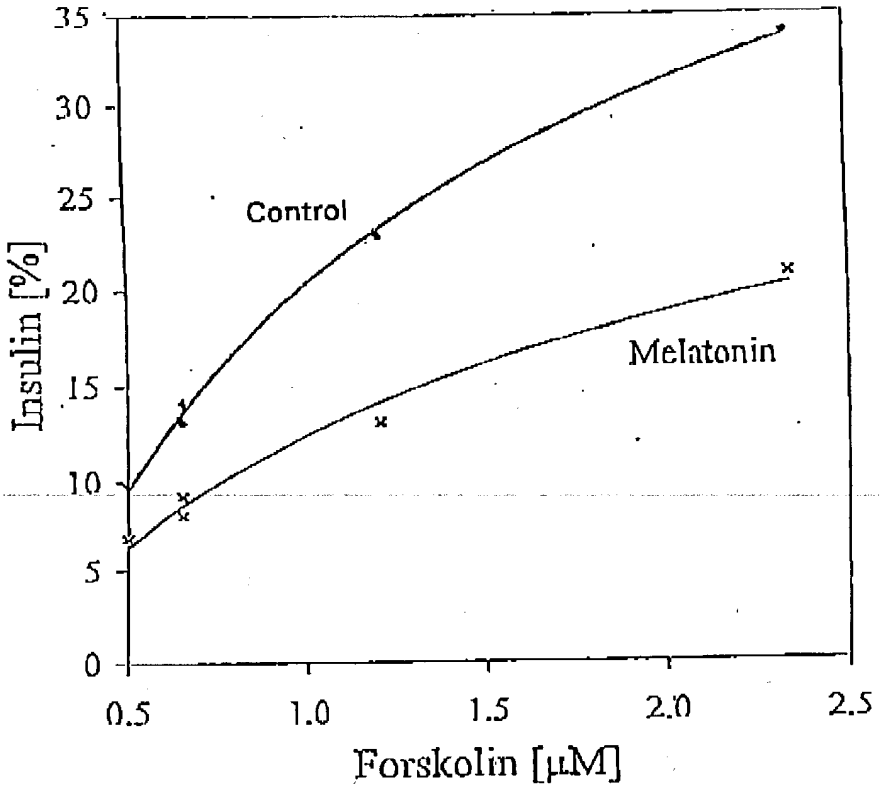
35
30
25
20
15
10
5
0
Insulin [%]
Control
Melatonin
0.5
1.0
1.5
2.0
2.5
Forskolin [µM]

Figure 2

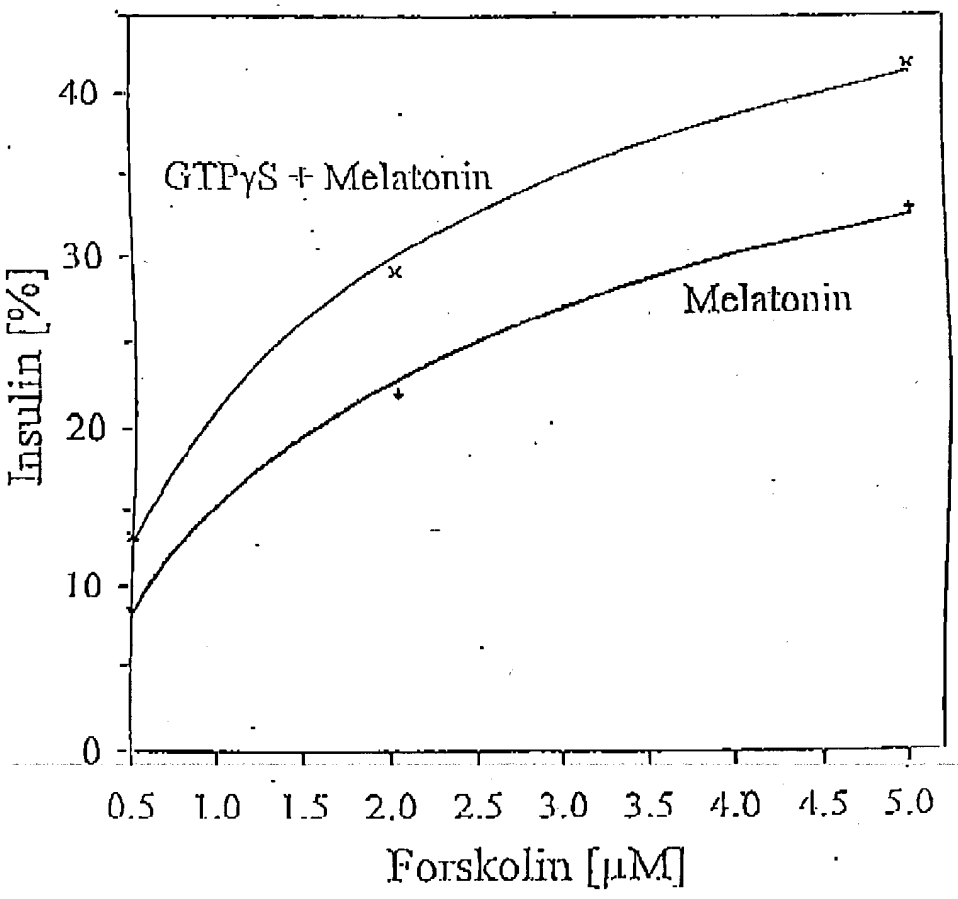
40
30
20
10
0
Insulin [%]
GTPγS + Melatonin
Melatonin
0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
Forskolin [μM]

Figure 3

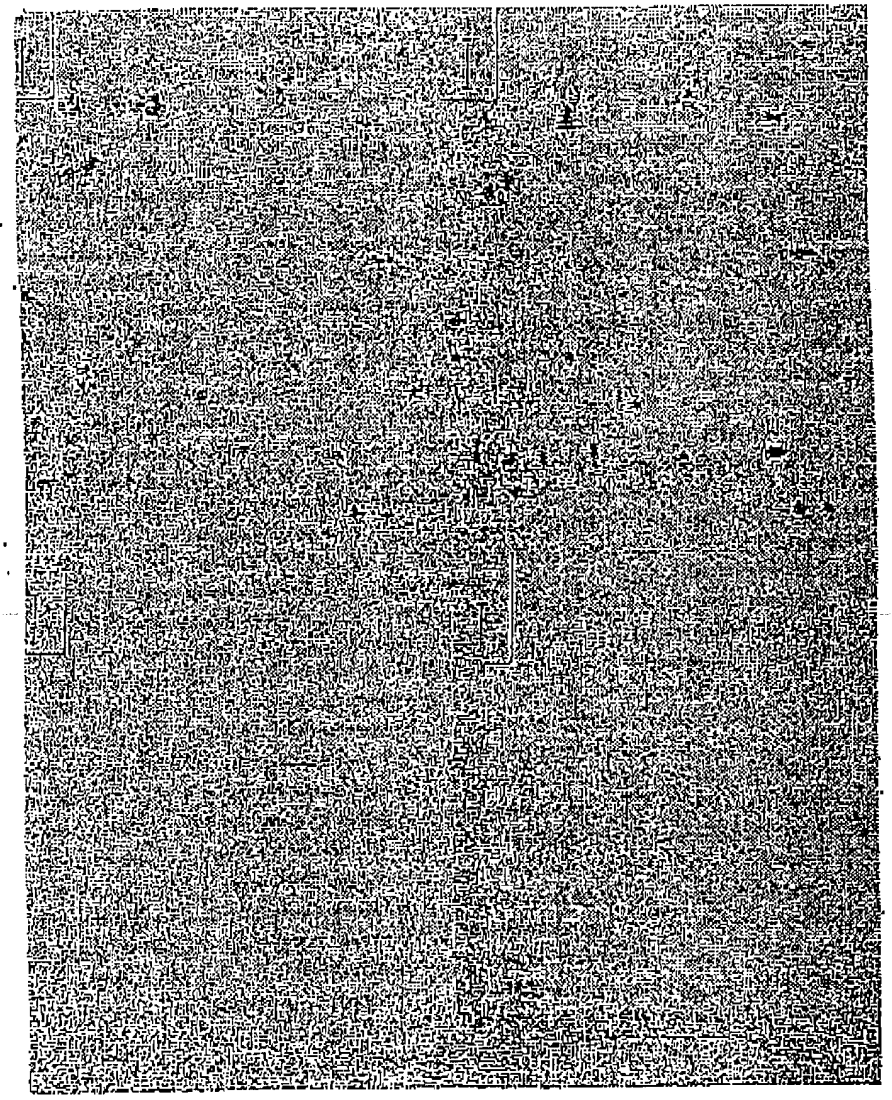

Figure 4

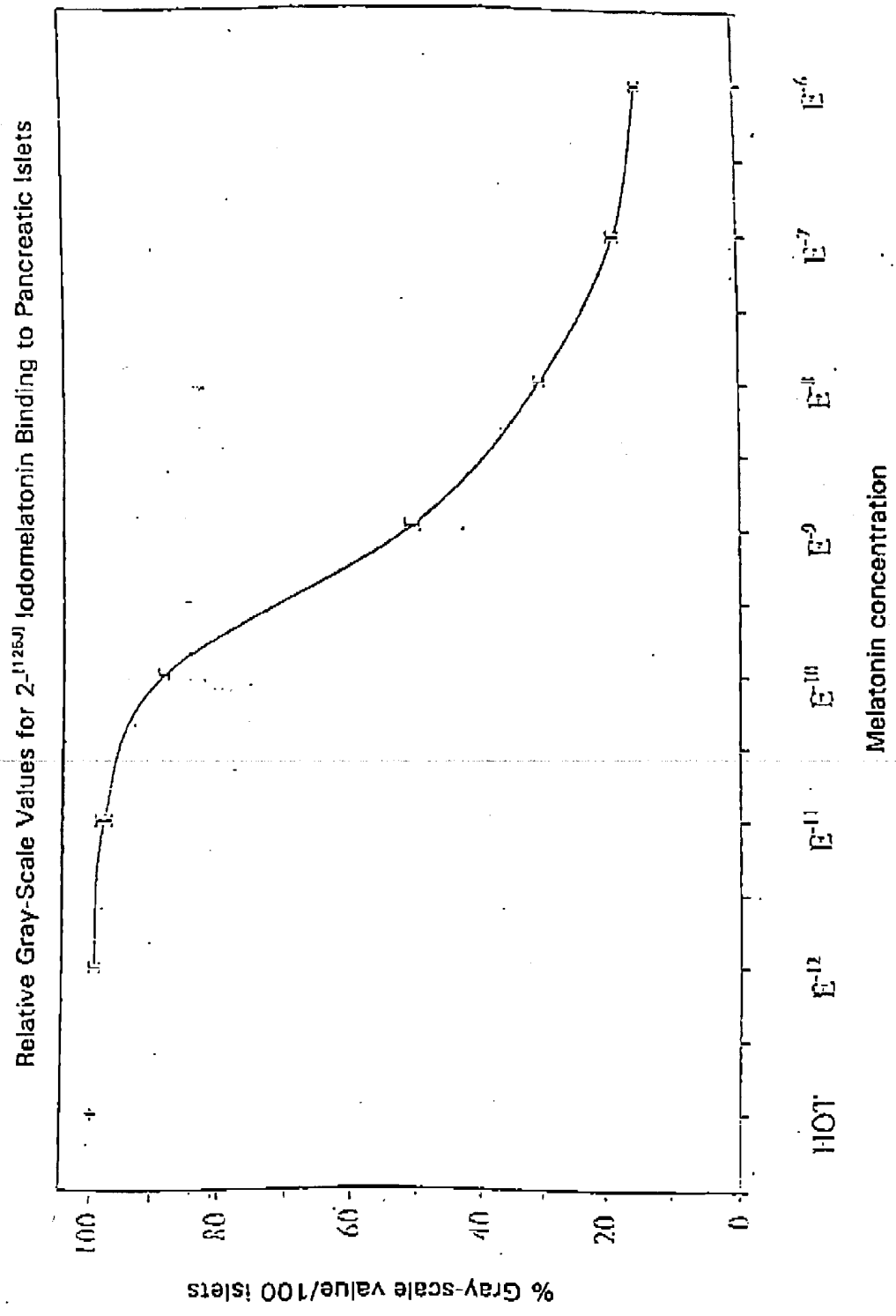
Relative Gray-Scale Values for 2-[125I] Iodomelatonin Binding to Pancreatic Islets
% Gray-scale value/100 islets
100
80
60
40
20
0
HOT
E-12
E-11
E-10
E-9
E-8
E-7
E-6
Melatonin concentration

Figure 5

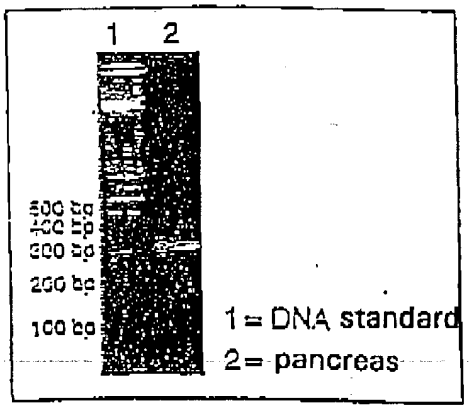

Figure 6